Investigation of the Experimental Limits of Small-Sample Heteronuclear 2D NMR

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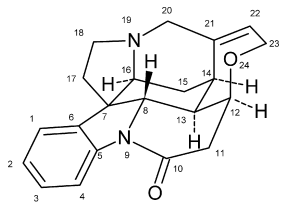
Practical experimental performance limits for an ensemble of heteronuclear 2D NMR experiments using a state-of-the-art 600 MHz 1.7 mm Bruker TCI Micro CryoProbe are reported. In the specific case of multiplicity-edited GHSQC, it was possible to acquire data on a 540 ng sample of strychnine (**1**; ∼1.6 nmol), prepared by serial dilution, which was used as a model compound. The experiments discussed also included GCOSY, ^{13}C reference spectra, ^{1}H−^{13}C GHMBC, IDR-GHSQC-TOCSY, 1,1-ADEQUATE, and ^{1}H−^{15}N GHMBC.

NMR spectroscopy, while capable of providing elegant atom-to-atom connectivity information that is vital to chemical structure characterization, is an inherently low-sensitivity technique when compared to optical spectroscopy or mass spectrometry. Over the past five decades considerable effort has been expended to improve magnet designs so that observation frequencies have risen correspondingly from 40 MHz to 1 GHz. Experiment design for two-dimensional heteronuclear shift correlation spectroscopy has likewise evolved from ^{13}C-detected methods to ^{1}H or "inverse-detected" methods with a consequent and significant improvement in sensitivity. Finally, probe designs were first inverted, i.e., the X-coil was moved outboard of the proton coil, followed by the development of successively smaller diameter probes.[1,2] Further significant gains in sensitivity were obtained by cooling the rf coils and preamplifiers to the range 20−25 K and ∼30−40 K, respectively.[3,4] Since 2007, Bruker BioSpin has offered commercially a 600 MHz TCI gradient inverse triple-resonance 1.7 mm MicroCryoProbe. As a result of a recent collaboration, a newly designed version of this probe with ^{15}N 90° pulse performance of ∼25 μs that is capable of exciting a 600 ppm bandwidth for ^{15}N at 60 MHz was installed in our laboratory.[5] We now report the results of an investigation into the performance limits of this probe technology for small-sample NMR investigations.

When evaluating the experimental performance of NMR probe technology, it is best to utilize a molecule that will allow a variety of experiments to be tested without having to change samples. Ideally, the model compound selected should be one that is well known to the investigators concerned and, to facilitate comparison work in other laboratories, a compound that is readily available. It was on this basis that we have utilized routinely the well-studied alkaloid strychnine (**1**),[6] the first alkaloid ever to be isolated in pure form[7] and one that was certainly a structural enigma to chemistry for more than 150 years.[8−10]



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An evaluation of the performance of a small-volume probe realistically needs to be done in reverse order. This has involved a consideration of the least sensitive experiments that have the highest sample requirements first, for example 1,1-ADEQUATE, and moving in the direction of the highest sensitivity experiments, e.g., ^{1}H reference spectra, which have the smallest sample requirements. Sample handling, of course, becomes increasingly important as sample sizes diminish.

Undoubtedly, the 1,1-ADEQUATE experiment will be the least sensitive experiment one might be likely to consider employing in the characterization of a small sample of an unknown compound.[11−13] A recent report by Cheatham and co-workers[13] notes that it is reasonable to be able to acquire a 1,1-ADEQUATE spectrum in a period of time ranging from a few hours to overnight on a few milligrams of material using a 5 mm cryogenic NMR probe. To test the feasibility of acquiring 1,1-ADEQUATE data using a 1.7 mm sample, a solution containing 870 μg (∼2.6 μmol) of strychnine/30 μL of deuterochloroform was prepared by serial dilution. A preliminary 1 h 50 min experiment showed that some of the expected correlations were observed,[14] prompting us to undertake an overnight (14 h 40 min) acquisition. A comparison of the 500 MHz data acquired using a 10 mg sample with those obtained using the 870 μg sample at 600 MHz using a 1.7 mm MicroCryoProbe is shown in Figure 1.

With the quantity of material used to acquire the 1,1-ADEQUATE spectrum, other 2D NMR experiments that one might wish to perform are all within easy experimental reach. The ensemble of experiments and acquisition times to obtain adequate signal-to-noise ratios include GCOSY, 7 min; ROESY, 1 h 11 min; ^{13}C reference, 25 min; multiplicity-edited ^{1}H−^{13}C, 7 min; ^{1}H−^{13}C GHMBC, 33 min; ^{1}H−^{13}C H2BC,[15,16] 3 h 11 min; ^{1}H−^{15}N GHMBC, 1 h 22 min; and IDR-GHSQC-TOCSY, 28 min.

Obviously, many samples of interest will not be as soluble as strychnine (**1**) in chloroform, nor will they necessarily be available in quantities of several micromoles. For this reason, we shifted from $CDCl_3$ to d_6-DMSO as a solvent and dropped the sample size to an initial 150 μg (0.45 μmol, prepared in d_6-DMSO by serial dilution). At this level, the full gamut of regularly employed 2D NMR experiments, with the exception of 1,1-ADEQUATE, which was not attempted, is still within easy reach. The most challenging of the experiments performed with the sample was an 8 Hz optimized ^{1}H−^{15}N GHMBC experiment, the results of which are shown in Figure 2. The initial data are shown in panel A of Figure 2 and were acquired in 2 h 13 min. All of the correlations to the N-9 amide resonance were observed,[17] and four of six possible correlations to the N-19 resonance were observed in these data. The slice through the F_2 dimension at the ^{15}N chemical shift of N-19 is superimposed on the spectrum. In this slice one may observe the weak correlation from H-15b and a hint of the correlation from the H-20b doublet. When the experiment was run overnight (12 h 25 min), the spectrum shown in panel B was obtained. All of the N-19 correlations were now well above the threshold of the contour

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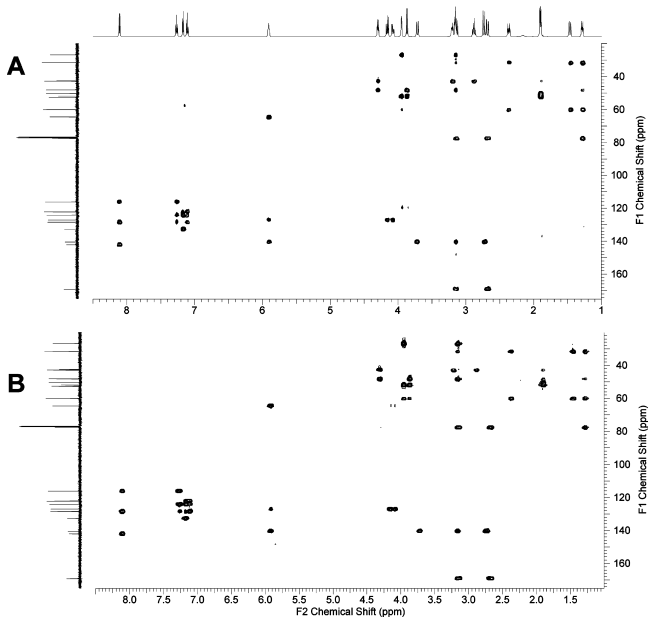

Figure 1. Comparative 1,1-ADEQUATE spectra of strychnine (**1**). (A) Results obtained using an 870 μg sample of **1** dissolved in 30 μL of deuterochloroform prepared by serial dilution. The data were acquired in 14 h 40 min using a Bruker 600 MHz NMR spectrometer equipped with a 1.7 mm MicroCryoProbe. (B) Results obtained using a 10 mg sample dissolved in ~200 μL of deuterochloroform in a 3 mm NMR tube. The data were acquired in 6 h 25 min using a Bruker 500 MHz NMR spectrometer equipped with a 5 mm TXI CryoProbe. Both experiments were optimized for a 60 Hz $^{13}C-^{13}C$ coupling.[13]

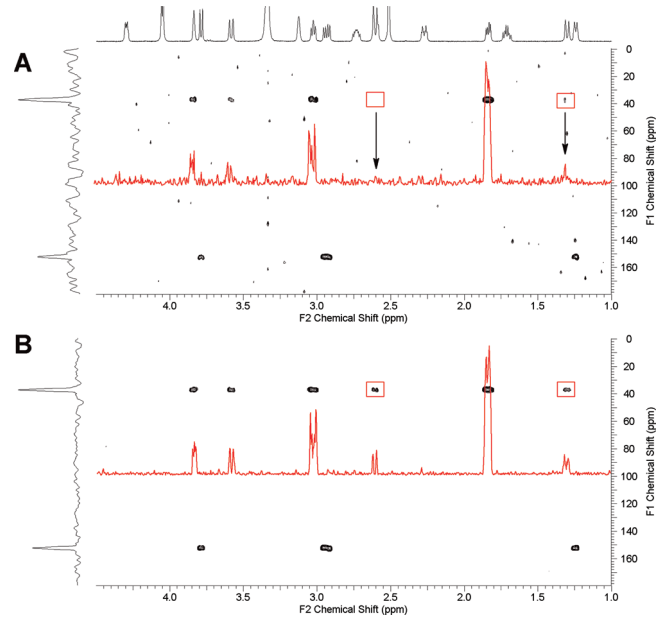

Figure 2. Strychnine (**1**) 8 Hz optimized $^{1}H-^{15}N$ GHMBC spectra recorded using a 150 μg sample of **1** in d_6-DMSO prepared by serial dilution. (A) Spectrum recorded in 2 h 13 min; the slice superimposed over the spectrum was taken at the N-19 chemical shift and shows the vestiges of the H-15b and H-20b correlations that are not yet observed (red boxed regions). (B) $^{1}H-^{15}N$ GHMBC spectrum of the same sample recorded overnight (12 h 25 min), in which all correlations are observed well above the threshold of the contour plot (compare red boxed regions for the two panels). For comparison, the slice at the chemical shift of N-19 is superimposed over the spectrum. Both contour plots are vertically flanked by the projection through the F_1 frequency domain showing the signal-to-noise ratio of the ^{15}N correlations.

plot (compare N-19 slice shown in red to that from the data in panel A). When dealing with an unknown molecule with a sample this size, the acquisition of $^{1}H-^{15}N$ GHMBC data should be considered as an overnight acquisition.

More routine 2D NMR experiments performed on the 150 μg sample included GCOSY, 15 min; $^{1}H-^{13}C$ multiplicity-edited GHSQC, 9 min; $^{1}H-^{13}C$ GHMBC, 33 min; and ROESY, 1 h 29 min. At this level, it was still possible to acquire a ^{13}C reference spectrum using the X-coil of the MicroCryoProbe, although the acquisition time for data with a satisfactory signal-to-noise ratio is in the range 18−36 h, as shown in Figure 3.

Working with still smaller samples, we anticipated that ^{13}C direct observe and $^{1}H-^{15}N$ GHMBC spectra would be the next casualties of sample size versus time constraints. However, with a 45 μg (~0.13 μmol) sample of strychnine (**1**) in 30 μL of d_6-DMSO prepared by serial dilution, it was still feasible to acquire an 8 Hz optimized long-range $^{1}H-^{15}N$ GHMBC spectrum over a weekend (see Figure S1, Supporting Information). After 13 h 30 min, the three most prominent responses in the spectrum were observed, but 49 h 30 min of data acquisition was required to see all of the possible long-range correlations to both nitrogen atoms. With the 45 μg sample of strychnine, GCOSY, GHSQC, and $^{1}H-^{13}C$ GHMBC were also readily acquired in 14 min, 9 min, and 4 h 30 min, respectively.

Serial dilutions below the 45 μg level became progressively more challenging in terms of preparing samples that were free of contaminant species. All glassware was handled on a single-use basis, and sample transfers were accomplished using fresh 24 G flexible Teflon needles attached to 50 μL Hamilton gastight syringes. The next serial dilution sample prepared was 23 μg (~0.07 μmol) of strychnine/30 μL of d_6-DMSO. It was possible to record a high-quality GCOSY spectrum in only 9 min. For a multiplicity-edited $^{1}H-^{13}C$ GHSQC spectrum, all of the aliphatic correlations were visible in a 14 min acquisition; good signal-to-noise, with all

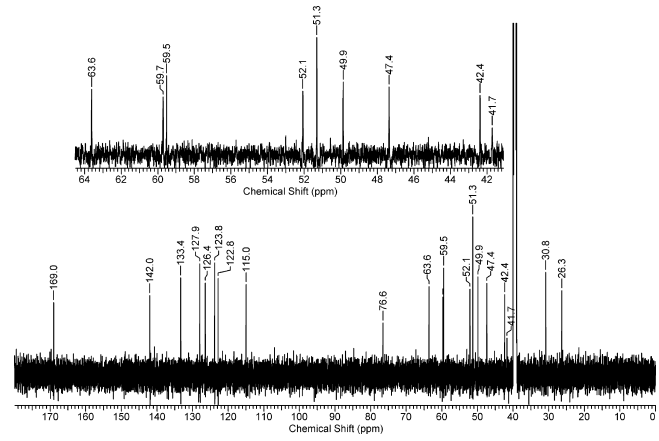

Figure 3. ^{13}C reference spectrum of 150 μg of strychnine (**1**) in d_6-DMSO recorded in 26 h 40 min using the X-coil of the MicroCryoProbe. The aliphatic region between DMSO and 65 ppm is expanded in the inset.

resonances well above the threshold of the contour plot, was achieved in 55 min (see Figure S2, Supporting Information). It is interesting to note that the H-22 vinyl resonance was not observed in the contour plot of the 14 min data and was defined only by a single contour in the 55 min spectrum. A good-quality $^{1}H-^{13}C$ GHMBC spectrum was recorded overnight on this sample.

A further serial dilution was prepared that afforded 5.4 μg/30 μL of d_6-DMSO (~0.016 μmol).[18] While a GCOSY spectrum could be recorded in 15 min with this sample, it was expected that the acquisition of heteronuclear shift correlation data on a sample of this size would provide a more significant challenge than the

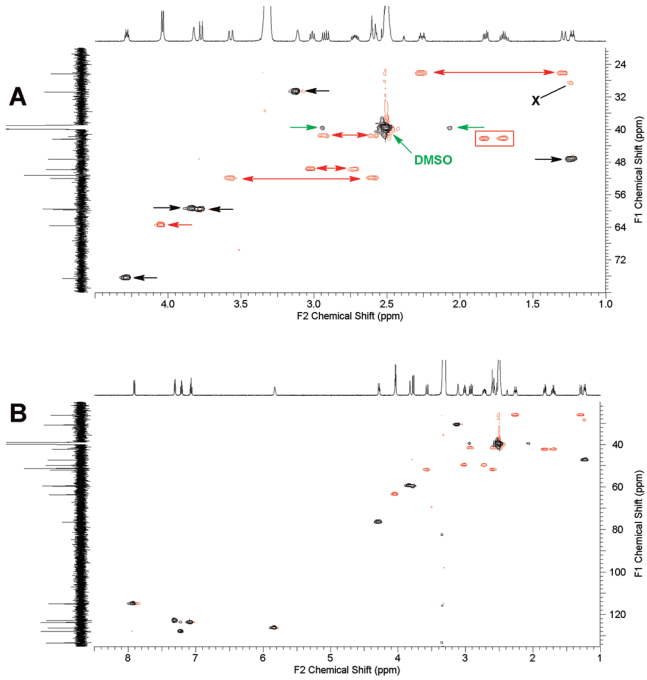

Figure 4. Multiplicity-edited $^{1}H-^{13}C$ GHSQC spectra of a 5.4 μg sample of strychnine (**1**) in d_6-DMSO prepared by serial dilution. (A) Expansion of the aromatic region of the spectrum. (B) Full spectrum. Methylene correlations are plotted in red and designated by single- or double-headed arrows or enclosed in a red box. Methine resonances are plotted in black and designated by single-headed arrows. The DMSO resonance is flanked by a pair of correlations designated by green arrows that are cycling side-bands due to the X-nucleus decoupling used to acquire the data.[19] These artifact responses are not observed in higher concentration data but were observed in all of the GHSQC spectra recorded using samples of ≤5.4 μg during the series of experiments performed.

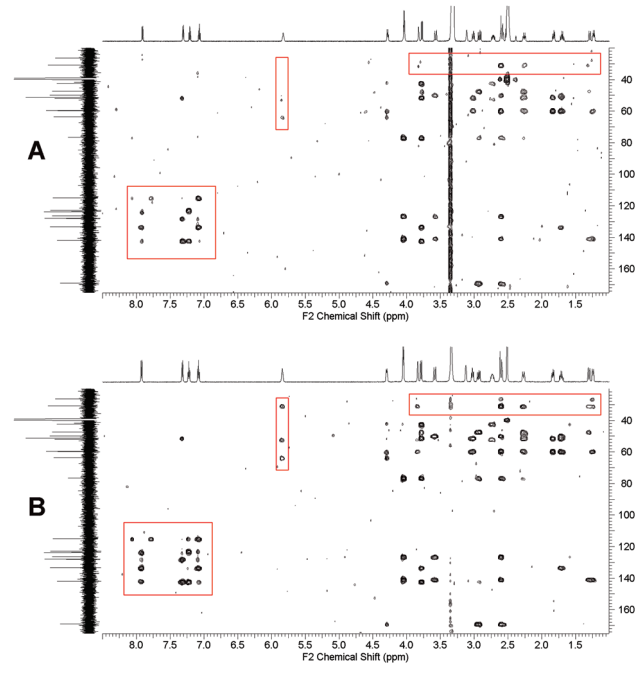

Figure 5. Comparison of the 8 Hz optimized 5.4 μg $^{1}H-^{13}C$ GHMBC spectrum of strychnine (**1**) recorded in 43 h 20 min (A) with the spectrum recorded using a 150 μg sample recorded in 33 min (B). While the data for the 5.4 μg sample shown in panel A are reasonably complete, there are some correlations to C-14 missing from the spectrum, as are all of the correlations to C-15. There are also two of the three correlations from the H-22 vinyl proton missing in panel A as well as some of the correlations within the aromatic region of the spectrum.

experiments described above. However, it was still possible to record a high-quality multiplicity-edited GHSQC spectrum overnight (14 h 45 min), which is shown in Figure 4. The expansion of the aliphatic region of the spectrum shown in panel A of Figure 4 showed, for the first time, cycling side-bands that arise as a consequence of the X-band decoupling scheme used (GARP) to acquire the data.[19] The 8 Hz optimized $^{1}H-^{13}C$ GHMBC spectrum on this sample was acquired over a weekend (43 h 20 min). While these data were generally of very good quality, some correlations that might have been observed in the spectrum were not. Specifically, correlations to the C-15 resonance were absent, and some of the long-range correlations in the aromatic region were missing, as were several of the correlations from the H-22 vinyl proton. Insofar as a 1.7 mm sample is concerned, the 5.4 μg (~0.016 μmol) sample used in this phase of the study may define a practical limit for the acquisition of $^{1}H-^{13}C$ GHMBC data. It is, however, possible that this threshold can be pushed still lower by utilizing a 1 mm tube run coaxially in a 1.7 mm MicroCryoProbe.

With the size of the samples that have been examined thus far, acquisition times are still manageable for $^{1}H-^{13}C$ heteronuclear shift correlation experiments. At still lower levels, however, the acquisition of test GHMBC spectra becomes prohibitively time-consuming, particularly when it might be expected to take up to a weekend to record a GHSQC spectrum. In contrast, GCOSY data can still be very quickly acquired even down into the submicrogram range.

A further serial dilution was performed, affording a sample that contained 1.75 μg of strychnine/30 μL of d_6-DMSO, confirmed by quantitative mass spectrometry. A GCOSY spectrum can be acquired with a sample of this size in which most of the vicinal correlations are observed in as little as 15 min. A spectrum in which the numerous long-range correlations are observed should be possible within a few hours. A comparison of the sensitivity-enhanced, multiplicity-edited GHSQC spectrum of the 1.75 μg sample overnight (Figure 6A, 18 h 25 min) and over a long weekend (Figure 6B, 73 h 30 min) versus a multiplicity-edited GHSQC spectrum acquired overnight with a 5.4 μg sample (Figure 6C, 14 h 40 min) is presented in Figure 6. All of the methylene correlations are still visible in the contour plot, although some are only slightly above the threshold of the contour plot in the overnight experiment. Clearly, the long weekend acquisition was overkill, and it is likely that a good quality spectrum with reliable response intensity for all resonances could be acquired during a normal weekend acquisition with a sample of this size.

The last serial dilution done produced a sample of 540 ng (~1.6 nmol).[18] The proton spectrum recorded in 20 transients on this sample is shown in Figure 7A (see Figure S3, Supporting Information). The multiplicity-edited GHSQC spectrum acquired in 59 h is shown in panel 7B. The majority of the resonances observed in the 73 h 30 min spectrum recorded on a 1.75 μg sample shown in panel 7C for comparison are observed in the 540 ng spectrum. As would be expected, a GCOSY spectrum was readily obtained on this sample.

In practical terms, with careful sample handling, working in a 1.7 mm tube with the Bruker 600 MHz TCI MicroCryoProbe used for this study, it is feasible to consider trying to record multiplicity-edited $^{1}H-^{13}$ C GHSQC spectra on samples of ~3 nmol when a full weekend of spectrometer time is available. When a sample of ~15 nmol is available, the acquisition time for a multiplicity-edited GHSQC spectrum decreases to an overnight run. With the same sample, a $^{1}H-^{13}C$ GHMBC spectrum is within experimental reach over a weekend. With samples in the range 50−75 nmol [20−25 μg of strychnine (**1**)], multiplicity-edited GHSQC becomes an almost trivial experiment, with these data accessible in a few hours

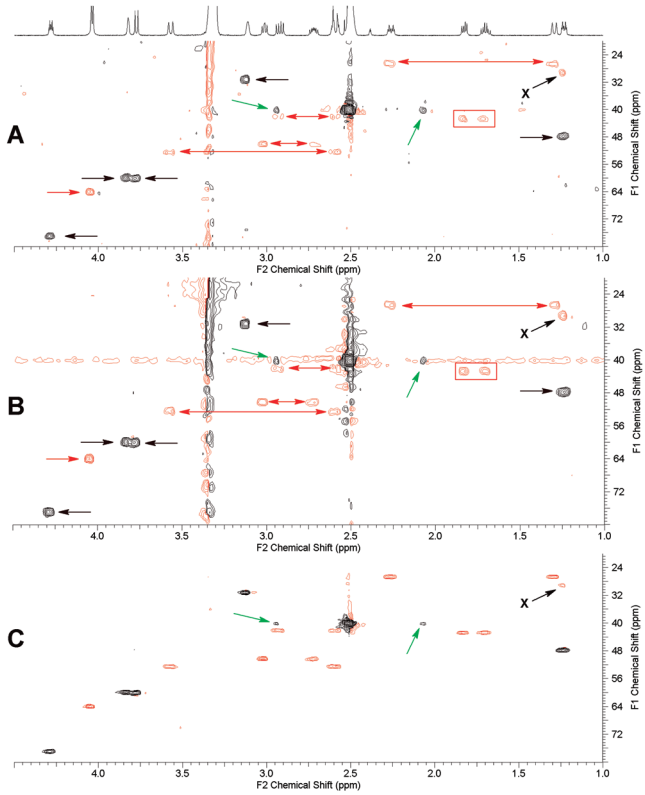

Figure 6. Comparative low-level multiplicity-edited $^{1}H-^{13}C$ GHSQC spectra of strychnine (**1**). Panels A and B were acquired using a sample containing 1.75 μg/30 μL, **1**. Panel C shows the same region of a spectrum acquired using a 5.4 μg/30 μL sample. Panel A was acquired as 2048 × 128 points with 320 transients/t_1 increment, giving an acquisition time of 18 h 25 min. Panel B was acquired with the same digitization but with 1320 transients/t_1 increment, giving an acquisition time of 73 h 30 min. In contrast, the data in panel C were acquired as 2048 × 256 points with 128 transients/t_1 increment, giving an acquisition time of 14 h 40 min. Methylene correlations are plotted in red; methine correlations are plotted in black. Correlations are denoted by single- or double-headed arrows or by a red box. Artifacts are denoted by "X". Cycling sidebands are denoted by green arrows.[19]

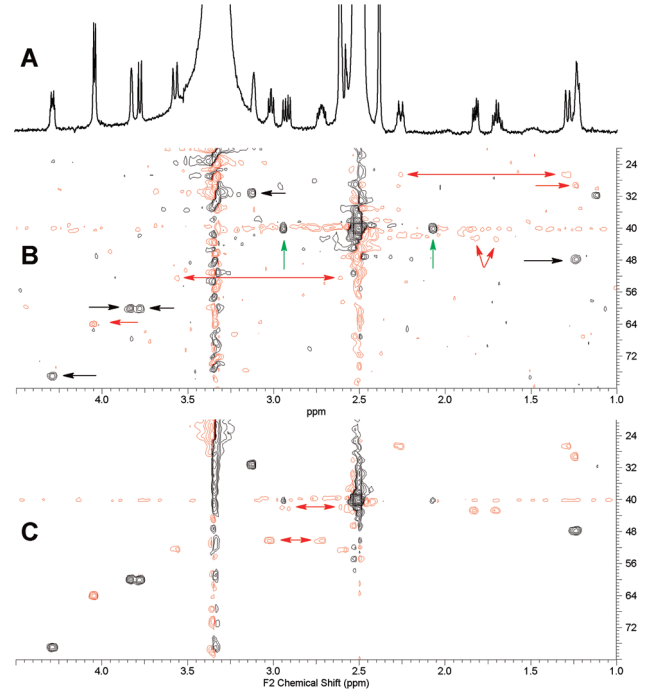

Figure 7. (A) ^{1}H reference spectrum acquired in 20 transients on a sample of 540 ng (~1.6 nmol) of strychnine (**1**) dissolved in d_6-DMSO prepared by serial dilution.[18] [The full proton spectrum is shown in Figure S3 (Supporting Information) with the ^{13}C satellites of the DMSO plotted on scale.] (B) Aliphatic region of the multiplicity-edited GHSQC spectrum of the 540 ng sample of **1** acquired in 59 h. (The protonated aromatic and 22-vinyl resonances all gave strong correlations as expected.) Methylene resonances are denoted by red single- or double-headed arrows. Methine resonances are denoted by black arrows. (C) Identical region of the multiplicity-edited GHSQC spectrum of the 1.75 μg sample recorded in 73 h 30 min (also shown in Figure 6B). Cycling side-bands arising from the GARP decoupling used for the X-nucleus are designated by green arrows.

at most. $^{1}H-^{13}C$ GHMBC likewise becomes a very reasonable experiment, requiring only ~4−8 h of spectrometer time for ample signal-to-noise. Continuing to still larger samples, $^{1}H-^{15}N$ GHMBC becomes accessible experimentally over a weekend with samples of ~125−150 nmol [~45 μg, ~150 nmol of strychnine (**1**)]. Multiplicity-edited $^{1}H-^{13}C$ GHSQC and GHMBC required 15 min and 4 h 30 min, respectively, for a sample of this size. Data acquisition times for a ~0.5 μmol sample of strychnine (**1**) decreased still further to less than an hour for both a multiplicity-edited $^{1}H-^{13}C$ GHSQC and GHMBC spectrum and overnight (12 h 25 min) for $^{1}H-^{15}N$ GHMBC data. Finally, with samples of several micromoles of **1**, it becomes possible to include the 1,1-ADEQUATE experiment in the experimental arsenal that can be employed to solve an unknown structure.

It is worth noting that with a 1 mg sample of strychnine (**1**, ~3 μmol) it is now possible to acquire the full gamut of homo- and heteronuclear 2D NMR experiments in 4 h (including $^{1}H-^{15}N$ GHMBC but not 1,1-ADEQUATE) that could, in principle, be used to establish the full chemical structure and stereochemistry of a molecule that proved such a structural enigma for chemists and originally took a century and a half to characterize structurally.[7−10]

Experimental Section

General Experimental Procedures. All experiments were performed on a Bruker Avance III three-channel 600 MHz NMR spectrometer equipped with a gradient inverse triple-resonance Bruker 1.7 mm TCI MicroCryoProbe. Standard NMR pulse sequences from the Bruker sequence library were uniformly employed for this study. Digitization in the F_2 and F_1 frequency domains was typically 2K points in the F_2 domain for GCOSY and ROESY experiments as well as the multiplicity-edited GHSQC and 1,1-ADEQUATE experiments. The $^{1}H-^{13}C$ and $^{1}H-^{15}N$ GHMBC spectra were digitized with 4K points in the F_2 domain; long-range delays were optimized for 8 Hz for the $^{1}H-^{13}C$ experimentes and 6 Hz for the $^{1}H-^{15}N$ experiments. Homonuclear 2D experiments employed 256 increments in the F_1 domain. The multiplicity-edited GHSQC and 1,1-ADEQUATE experiments employed 160 increments in the F_1 domain. Finally, the $^{1}H-^{13}C$ GHMBC experiments were digitized by 160 or 192 increments in the F_1 domain; the $^{1}H-^{15}N$ GHMBC experiments were digitized with 96 increments in the F_1 frequency domain. All samples were prepared by serial dilution in volumetric glassware using solvents provided by Cambridge Isotope Laboratories in glass ampules. Samples of 30 μL were transferred to 1.7 mm Bruker NMR tubes using fresh 24 G Teflon needles and a Hamilton 100 μL gastight syringe. All samples prepared in which the expected total material in the sample was <10 μg had their concentrations verified using quantitative mass spectrometry to ensure validity.

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Supporting Information Available: Selected low-level NMR spectra are available free of charge via the Internet at http://pubs.acs.org.

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